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QUARTERLY

Minireview

Methods of peptide conformation studies★

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In solution most of the peptides assume multiple flexible conformations. Determination of the dominant conformers and evaluation of their populations is the aim of peptide conformation studies, in which theoretical and experimental methods play complementary roles. Molecular dynamics or Monte Carlo methods are quite effective in searching the conformational space accessible to a peptide but they are not able to estimate, precisely enough, the populations of various conformations. Therefore, they must be supplemented by experimental data.

In this paper, a short review of the experimental methods, most widely used in peptide conformational studies, is presented. Among them NMR plays the leading role. Valuable information is also obtained from hydrogen exchange, fluorescence resonance energy transfer, and circular dichroism measurements. The advantages and shortcomings of these methods are discussed.

Conformational studies of peptides are aimed at two goals. The first one, historically earlier, is to learn about the conformational properties of polypeptide chains and to determine factors that govern the process of protein folding and stabilize the native structure of a protein [1]. The most striking examples of the achievements in this field are: the discovery of the α-helix and β-sheet structures from an analysis of crystal structures of short

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Abbreviations: CD, circular dichroism; FRET, fluorescence resonance energy transfer; MC, Monte Carlo; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy.

model peptides [2, 3], and the elucidation of the α-helix formation process (the so called helix-coil transition) from extensive studies of a series of model peptides with strong propensity for the α-helical structure [4].

Recently, numerous peptide conformation studies have been carried out with the aim to elucidate the phenomenon of protein misfolding responsible for amyloid diseases and to design peptide molecules that possibly could be used to prevent amyloid precipitation [5]. The conformational properties of β-amyloid peptide and of the structures of its deposits responsible for Alzheimer disease are also studied very extensively [6].

Another goal of peptide conformation investigations is to determine the relationship between conformation and activity of biologically important peptides, e.g. hormones. Such studies are usually application-oriented with the ultimate aim to design potent and very selective enzyme inhibitors and receptor blockers that could be used in pharmacology [7].

The beginning of peptide conformational studies in solution date back to early 1950s. The results obtained at that time using rather rough methods such as optical rotary dispersion (ORD), viscosity, and sedimentation measurements led to the conclusion that, at least in water, the conformation of linear peptides is extremely flexible and can be described by the "random coil" model. According to this model, every amino-acid residue, independently of its neighbors, randomly probes the conformational space sterically available to it. Only much later, at the end of 1970s, with the accumulation of a large amount of data provided by high resolution NMR, fluorescence and circular dichroism spectroscopy, it became evident that the conformation of even short, linear peptides is far from random. In fact, a vast majority of the conformations available to a peptide is only marginally populated and very often the population of just a few of them is very high. For example, it was shown that at favorable conditions about 35% of 13-residue long C-peptide of RNase A assumes the α-helical conformation [8]. As much as 70% of the *cis* isomer of the peptide AYPYDV was found in the β-turn conformation [9].

Numerous biological experiments strongly support the results of conformational investigations, clearly indicating that peptide activity is determined not only by the presence of functionally active groups that bind to a target protein but also depends dramatically on the conformational properties of the whole peptide.

Nevertheless, apart from some special systems (such as metal-saturated metallopeptides, e.g. the calcium binding loops of EF-hand proteins [10] or zinc fingers [11]) even relatively rigid, cyclic peptides typically assume multiple conformations. Moreover, these conformations are rather flexible, with torsional angles of the backbone (φ, ψ) as well as of the side chain groups (χ_i) fluctuating within large intervals. Therefore, peptide conformation must be described in terms of the relative population of the dominant conformers. Information about dynamical properties of these conformers is also desirable. Consequently, a full description of peptide conformation is possible only using theoretical methods, such as molecular dynamics (MD) or Monte Carlo (MC) simulations. These methods provide effective tools for searching the conformational space accessible to the peptide molecule but they are not able to evaluate the free energies of various conformations with a precision grater than about 2 kcal/mol, at best, so that the calculated conformer populations may differ from real ones by two or more orders of magnitude. Therefore, the population estimates must be based on experimental data.

NUCLEAR MAGNETIC RESONANCE (NMR)

The basic structural information provided by NMR comes from interproton nuclear

Overhauser effect (NOESY or ROESY spectra) and assumes the form of the so-called "contact map" indicating the pairs of protons that are close to one another in space; closer than about 6 Å. In addition, coupling constants between NH and CαH protons contain information about the average values of the peptide backbone torsional φ angles. The restraints derived from these data make it possible to determine protein structures *via* restrained molecular dynamics calculations. This method is, nevertheless, of little value in the case of peptides that populate multiple ensembles of conformations characterized by different sets of restraints.

A number of methods have been proposed to solve this problem [12]. In our lab the "maximum entropy approach" proposed by Groth *et al.* [13] is used [14]. The calculations consist of the following steps. 1) The conformational space of a peptide is searched using the electrostatically driven MC method to find conformations with reasonably low energies. 2) The calculated conformations are clustered into families of similar conformers. 3) For each family a NOESY spectrum is generated and NH-CαH coupling constants are calculated. 4) The populations of the families are determined by fitting a linear combination of the generated spectra and coupling values to the experimental data*. The results obtained in our lab for a series of cyclic enkephalin analogues were fully consistent with biological activity tests [15].

Nevertheless, it should be stressed here that peptide conformations derived from NMR data are not as trustworthy as NMR protein structures. Peptide molecules are much less compact and, consequently, their NOESY spectra show relatively few and weak contacts, particularly the most informative, long-range ones. It can always happen that some conformations do not show any contacts and are missed. On the other hand, a false assignment of even a single contact can lead to a dramatic overestimation of the population of a particular conformation.

NMR can also be used to study peptide association. The association equilibrium constants can be determined from the analysis of concentration-dependent chemical shifts of the peptide proton signals and, if at medium concentrations the association rate is close to the association-induced frequency shift $\Delta\omega$, the rate constant of the association process can be determined from the line shape analysis [16].

Apart from hydrophobic interactions, hydrogen bond formation is the most important factor stabilizing the conformation of a polypeptide chain. Therefore, the information which hydrogen bonds are formed and to what extent they are populated is of uttermost importance in the investigation of peptide conformation.

The backbone NH…O=C bonds can be identified from $^{3h}J_{NC'}$ couplings between ^{15}N and ^{13}C nuclei. The hydrogen bond populations can be estimated from the coupling constants. Nevertheless, only highly populated hydrogen bonds can be detected using this method. Therefore, it is rarely applicable in the peptide studies [17] although it can provide extremely valuable data concerning protein structures [18, 19].

A partial information about hydrogen bonds in peptides can be obtained from hydrogen-exchange experiments. The populations of hydrogen bonds formed by individual NH groups can be estimated, albeit the proton acceptor groups cannot be identified in this way. The NMR techniques used in hydrogen exchange experiments will be presented in the next chapter.

*Using the method of Groth *et al.* [13] any kind of conformational data can be analyzed; not only those coming from NMR measurements but also provided by hydrogen exchange or FRET experiments (see below). Therefore, a global data analysis can be performed.

HYDROGEN EXCHANGE

In aqueous solutions the free amide groups of the peptide backbone exchange their protons with water molecules. The exchange rate constants depend on temperature, pH, and on the peptide sequence. In an unordered, coil conformation only the side chains of the residues forming a particular peptide group affect, in an additive way, the exchange rate of the group's NH proton, whereas the influence of the other residues can be neglected.

The effects of all twenty amino-acid side chains positioned on the left and right side of the peptide group have been determined and the temperature and pH dependence of the exchange rates has been established [20]. Also, the corrections due to isotopic effects in H→D and D→H exchange processes have been determined [21]. From these data the exchange rate k_c can be calculated for any individual amide group of a peptide in the coil conformation.

The protons involved in hydrogen bonds are protected against the exchange. Therefore, if an NH group is partially engaged in hydrogen bonding the exchange rate of its proton, k_{ex}, is smaller than k_c. The protection factor:

$$P = k_{ex}/k_c \qquad (1)$$

is directly related to the population x of the hydrogen bond(s):

$$x = (P - 1)/P \qquad (2).$$

At low temperatures (about 0°C) and pH ≈ 3, the exchange process is slow enough to be monitored directly by measuring, in consecutive time intervals, one-dimensional ^1H NMR spectra of deuterated peptide dissolved in H_2O or of the protonated one, dissolved in D_2O.

At neutral pH the exchange rates are comparable to those of nuclear magnetic longitudinal relaxation of the NH peptide protons (about 1 s^{-1}) and can be determined from NMR magnetization transfer experiments [22].

Until recently mass spectroscopy has been used only to characterize, in a global way, the H → D exchange without measuring the exchange rates of individual peptide hydrogens [23, 24]. Nevertheless, it has been demonstrated that such measurements can be done using Fourier transform ion cyclotron resonance mass spectrometry [25]. Also, it seems quite feasible to monitor the H → D exchange of individual hydrogens by sequencing the peptides in a tandem quadrupole – time of flight MS instrument.

The advantages of MS spectroscopy, as compared with NMR, are numerous. At least one thousand times less material is needed for the experiment. Not only slow exchange rates (k_{ex} < 0.1 min^{-1}) and those close to 1 s^{-1} can be measured, but all of them up to 10 s^{-1}, if a stopped-flow device is used to mix peptide solution with D_2O and to quench the exchange reaction by shifting pH to about 3. Moreover, very often ^{15}N-labeled peptides must be used in NMR experiments to separate the overlapping NH proton signals by measuring the ^{15}N-edited spectra.

The main source of errors in the determination of hydrogen bond populations using the hydrogen exchange technique is that the exchange rate constants of free NH groups of the peptide need not necessarily be equal to k_c values. Consequently, the calculated protection factors may not be correct.

From our experiments (unpublished data) it follows that in some conformations peptide amide protons can be considerably protected against the exchange (1 < p < 4) even if they are not engaged in hydrogen bonds. Most probably this phenomenon is due to the formation of hydrophobic clusters of the peptide side chain groups. On the other hand, in some conformations a particular amide hydrogen can be more exposed to the solvent and exchange faster (0.5 < p < 1) than in the coil.

These effects can lead to a considerable over- or under-estimation, respectively, of the hydrogen bond populations.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

The energy transfer between two chromophores – an excited donor and an acceptor in its ground state – on distances exceeding 5 Å is described by Förster's theory [26]. The transfer rate constant k_T equals:

$$k_T = (R_0/r)^6/\tau_D \qquad (3)$$

where r is the distance between the chromophores, τ_D – the donor fluorescence lifetime in the absence of the acceptor, and R_0 – the critical distance characteristic for each acceptor and donor pair and dependent on their mutual orientation.

Since k_T, as it follows from Eqn. 3, depends very strongly on r, it can be used as a "spectroscopic ruler" to measure interchromophoric distances with a precision comparable to that of the X-ray or NMR methods.

In the case of peptide studies the experimental procedure is as follows. First of all, a pair of chromophores must be introduced into specific sites of the peptide molecule. Sometimes one of the natural peptide chromophores, i.e. tryptophane or tyrosine, can be used as the donor. The R_0 value of the donor-acceptor pair should be close to the expected distance between them, because only the distances within the interval of ±50% of R_0 can be measured with a satisfactory precision. On the other hand, r must be greater than about 5 Å because at shorter distances the energy transfer is dominated by the exchange (Dexter) mechanism and Förster's theory is no longer valid.

A large selection of chromophore pairs can be found in the literature (see refs. given in [27]) with R_0 values determined assuming random mutual orientation of the chromophores. This assumption almost always holds true for peptides, because of their conformational flexibility. It is worth to note that, because R_0 depends on τ_D, it can be down-regulated by adding external quenchers to the peptide solution [28, 29].

Several methods can be used to study the energy transfer process. The most informative are time-resolved measurements. If only one conformation is populated, with a strictly fixed distance between the chromophores, the fluorescence decay of the donor is monoexponential and its time constant τ_{DA} equals:

$$\tau_{DA} = \tau_D/[(R_0/r)^6 + 1] \qquad (4).$$

If the conformation is flexible and the donor-acceptor distance assumes various values a multiexponential fluorescence decay is observed. An analysis of the decay time constant distribution can be made and the results transformed into a distance distribution according to Eqn. 4.

Finally, if there are various conformations, with different average r values, a separate lifetime distribution of τ_{DA} is observed for each of them. The integrals of the distribution functions are proportional to the populations of the conformers. An example of fluorescence lifetime distributions clearly indicating the presence of three different peptide conformations is shown in Fig. 1.

Two factors determine the shape of the τ_{DA} distribution functions. One of them is the static distribution of interchromophoric distances. The other one arises from diffusional chromophore movements characterized by relaxation times comparable to, or shorter than, the average donor fluorescence time. Under the assumption of the Gaussian or the Lorentzian distribution of the static r distances, it is possible to calculate the rates of the mutual diffusion of the donor and acceptor groups from the analysis of the τ_{DA} distribution function [30]. It remains an open question whether these data reflect the conformational flexibility of the peptide itself or describe only the local mobility of the chromo-

phores. Nevertheless, if the diffusional effects are not taken into account the calculated distributions of distances are distorted and their average value can be significantly underestimated.

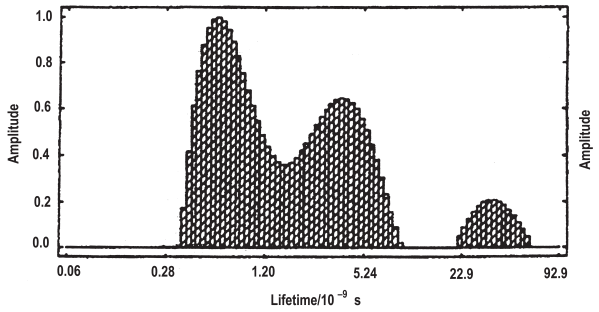

Figure 1. Fluorescence lifetime distributions of β-naphtylalanine in Phe(p-NO_2)-*cyclo*(Dab-Pro-Nal-Leu) — a cyclic enkephalin analogue.

Reproduced from [34] with the permission of the authors.

FRET is a method providing valuable, unique, and precise information about peptide conformation and dynamics. Its main drawback is that chemical modifications, i.e. introduction of the donor and the acceptor, may considerably perturb, in an unpredictable way, the peptide conformational properties.

CIRCULAR DICHROISM (CD)

Because of its simplicity, CD spectroscopy is widely used to determine the secondary structures of proteins [31]. Within the UV region from 180 up to 240 nm each of the structures: α-helices, β-sheets, and the remaining, unordered part of the polypeptide backbone, usually referred to as the "random coil", contribute in different ways to the protein spectrum. These contributions, viz. the spectra characteristic for particular secondary structures, have been calculated from comparative analysis of CD spectra of some polypeptides or reference proteins with known X-ray structures. An example of a set of such secondary structure spectra is shown in Fig. 2. By fitting a linear combination of them to the experimental spectrum the percentage of peptide groups engaged in various secondary structures can be calculated.

A number of programs for the analysis of CD data are available in the literature. They use various mathematical approaches and different sets of secondary structure spectra derived from CD measurements of various collections of reference structures. Sometimes, the contribution of the β-turn spectrum is also calculated.

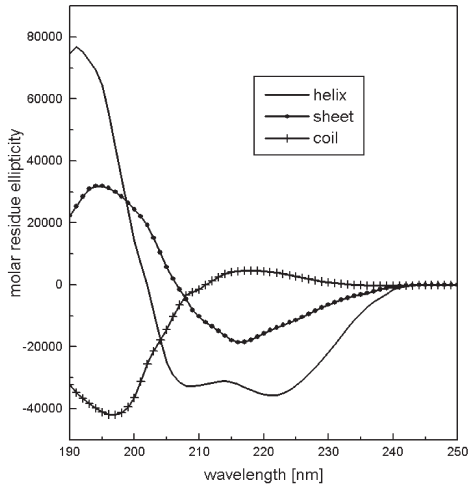

Figure 2. The reference spectra of the α-helix, β-sheet and "random coil" structures [35] used by the CDFIT program for the analysis of protein CD data.

B. Rupp, 1996, http://www-structure.llnl.gov/cd/cdtutorial.htm#Program.

For numerous reasons, only a rough estimation of the protein secondary structure is possible in this way. The CD spectrum of the polypeptide backbone is distorted by unknown contributions of phenylalanine, tyrosine, and tryptophane side chains, as well as of disulfide bonds, if present. The unordered segments of the protein backbone are far from being random, but they are fixed in more or less well-defined and rigid structures with unknown CD spectra. Only in large proteins they become statistically randomized. The intensity of the CD spectra of α-helices is length-de-

pendent and the contribution of very short segments, containing up to four peptide groups in the helical structure, is negligible [32]. Moreover, usually the measurements within the region from 180 up to 200 or even 210 nm cannot be done because of a strong solution absorbance. The use of low buffer concentrations and low absorbing buffers, such as potassium phosphate, along with sodium or potassium fluorides, instead of chlorides, to maintain the desired salt concentration, is often impossible. Consequently, only data limited to the region of about 205–240 nm are available for analysis.

Quantitative analysis of peptide CD data is even less reliable than in the case of proteins and often leads to quite erroneous conclusions. In fact, it is justifiable only in the studies of the equilibrium between two conformations with well known CD spectra. E.g., the intensity of the CD signal at 222 nm is widely used as a measure of the α-helix content in the helix-coil transition studies of model peptides [32, 33].

On the other hand, CD can be a sensitive, qualitative tool for monitoring peptide conformational changes induced by pH, temperature, salt concentration, etc. In particular, if the spectrum changes noticeably with increasing urea concentration, the peptide conformation is certainly not random. The opposite is not always true. A combination of a few specific, highly populated conformers can give rise to a spectrum closely similar to that of the "random coil". Unfortunately, CD theory is not sufficiently well developed to allow simulations of the spectra of given polypeptide chain conformations.

Although simple, CD measurements need experience and care. They are plagued by errors due to erroneous background corrections and distortions of the short-wavelength spectrum regions recorded at a too low signal to noise ratio.

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